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Propensity of red blood cells to undergo P2X7 receptor-mediated phosphatidylserine exposure does not alter during in vivo or ex vivo aging

Reece A. Sophocleous
University of Wollongong, rs256@uowmail.edu.au

Phillip R.F Mullany
University of Wollongong, pm563@uowmail.edu.au

Kelly M. Winter
Australian Red Cross Blood Service

Denese C. Marks
Australian Red Cross Blood Service

Ronald Sluyter
University of Wollongong, rsluyter@uow.edu.au

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Abstract

Phosphatidylserine (PS) exposure facilitates the removal of red blood cells (RBCs) from the circulation, potentially contributing to the loss of stored RBCs after transfusion, as well as senescent RBCs. Activation of the P2X7 receptor by extracellular adenosine 5'-triphosphate (ATP) can induce PS exposure on freshly isolated human RBCs, but whether this process occurs in stored RBCs or changes during RBC aging is unknown. STUDY DESIGN AND METHODS RBCs were processed and stored according to Australian blood banking guidelines. PS exposure was determined by annexin V binding and flow cytometry. Efficacy of P2X antagonists was assessed by flow cytometric measurements of ATP-induced ethidium+ uptake in RPMI 8226 cells. Osmotic fragility was assessed by lysis in hypotonic saline. RBCs were fractionated by discontinuous density centrifugation. RESULTS ATP (1 mmol/L) induced PS exposure on RBCs stored for less than 1 week. This process was near-completely inhibited by the P2X7 antagonists A438079 and AZ10606120 and the P2X1/P2X7 antagonist MRS2159 but not the P2X1 antagonist NF499. ATP-induced PS exposure on RBCs was not dependent on K+, Na+, or Cl− fluxes. ATP did not alter the osmotic fragility of stored RBCs. ATP-induced PS exposure was similar between RBCs of different densities. ATP-induced PS exposure was also similar between RBCs stored for less than 1 week or for 6 weeks. CONCLUSION The propensity of RBCs to undergo P2X7-mediated PS exposure does not alter during in vivo and ex vivo aging. Thus, P2X7 activation is unlikely to be involved in the removal of senescent RBCs or stored RBCs after transfusion.

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Propensity of red blood cells to undergo P2X7 receptor-mediated phosphatidylserine exposure does not alter during *in vivo* or *ex vivo* aging

Running head: P2X7-mediated PS exposure on aged RBCs

Authors: Reece A. Sophocleous,[1,2,3]* Phillip R.F. Mullany,[1,2,3]* Kelly M. Winter,[4] Denese C. Marks,[4] and Ronald Sluyter[1,2,3]

Institutions: [1]School of Biological Sciences, University of Wollongong, Wollongong, NSW, Australia; [2]Centre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW, Australia; [3]Illawarra Health and Medical Research Institute, Wollongong, NSW, Australia; and [4]Research and Development, Australian Red Cross Blood Service, Sydney, NSW, Australia.

Disclaimers: *Both contributed equally as first authors.

Address for correspondence: Ronald Sluyter, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia. Tel: +61-2-42215508. FAX: +61-2-42218130. e-mail: rsluyter@uow.edu.au.

Address reprint request to: Ronald Sluyter, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia. e-mail: rsluyter@uow.edu.au.

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Abbreviations: ATP = adenosine 5'-triphosphate; EC_{50} = half maximal effective concentration; FITC = fluorescein isothiocyanate; IC_{50} = half maximal inhibitory concentration; MFI = mean fluorescence intensity; NMDG = N-methyl-D-glucamine; PS = phosphatidylserine; RBC = red blood cell; SAGM = saline adenine glucose mannitol.

ABSTRACT

BACKGROUND: Phosphatidylserine (PS) exposure facilitates the removal of RBCs from the circulation, potentially contributing to the loss of stored RBCs following transfusion, as well as senescent RBCs. Activation of the P2X7 receptor by extracellular adenosine 5'-triphosphate (ATP) can induce PS exposure on freshly isolated human RBCs, but whether this process occurs in stored RBCs or changes during RBC aging is unknown.

STUDY DESIGN AND METHODS: RBCs were processed and stored according to Australian blood banking guidelines. PS exposure was determined by Annexin V binding and flow cytometry. Efficacy of P2X antagonists was assessed by flow cytometric measurements of ATP-induced ethidium$^+$ uptake in RPMI 8226 cells. Osmotic fragility was assessed by lysis in hypotonic saline. RBCs were fractionated by discontinuous density centrifugation.

RESULTS: ATP (1 mM) induced PS exposure on RBCs stored for less than 1 week. This process was near-completely inhibited by the P2X7 antagonists, A438079 and AZ10606120, and the P2X1/P2X7 antagonist, MRS2159, but not the P2X1 antagonist, NF499. ATP-induced PS exposure on RBCs was not dependent on K^+, Na^+ or Cl^- fluxes. ATP did not alter the osmotic fragility of stored RBCs. ATP-induced PS exposure was similar between RBCs of different densities. ATP-induced PS exposure was also similar between RBCs stored for less than 1 week or for 6 weeks.

CONCLUSION: The propensity of RBCs to undergo P2X7-mediated PS exposure does not alter during *in vivo* and *ex vivo* aging. Thus, P2X7 activation is unlikely to be involved in the removal of senescent RBCs or stored RBCs following transfusion.

Keywords: erythrocyte, blood storage, red cell storage lesion, purinergic receptor, purinergic signaling.

INTRODUCTION

During storage red blood cells (RBCs) undergo a series of biochemical and structural changes, which are collectively termed the red cell storage lesion, and are associated with reduced quality and lifespan of RBCs during storage and following transfusion.[1] These changes include increased exposure of phosphatidylserine (PS) on the RBC plasma membrane,[2] which parallels changes in RBCs aging *in vivo.*[3] Collectively, these and other studies support the concept that RBCs continue to age during *ex vivo* storage.[4] PS exposure may facilitate the removal of RBCs from the circulation by splenic and liver macrophages, potentially contributing to the loss of stored RBCs following transfusion, as well as senescent RBCs and RBCs in disease states such as sickle cell anemia and thalassemia.[5] Thus, there is continued interest in identifying the mechanisms contributing to PS exposure on RBCs aged *in vivo* and *ex vivo*.

Various stress treatments can induce PS exposure on freshly isolated and stored RBCs.[6,7] Moreover, stored RBCs show an increased susceptibility to undergo stress-induced PS exposure, which may be responsible for the increased loss of RBCs following transfusion.[6] Our group has shown that extracellular adenosine 5'-triphophate (ATP), via the activation of the ATP-gated cation channel P2X7, can also induce PS exposure on freshly isolated human RBCs.[8,9] Whether P2X7 activation can also induce PS exposure on stored RBCs and whether such cells following extended storage display an increased propensity to undergo ATP-induced PS exposure is not known. Human RBCs also express P2X1,[10,11] which along with P2X7 belong to the P2X family of purinergic receptor channels.[12] However the contribution of P2X1 activation to ATP-induced PS exposure remains unknown. Finally, in contrast to stress-induced PS exposure on RBCs,[7] P2X7-induced PS exposure on these cells does not require Ca^{2+} influx and does not coincide with cell shrinkage,[9] however the mechanism by which P2X7 mediates PS on RBCs remains to be elucidated.

Therefore, the aim of the study was to establish whether incubation with extracellular ATP could induce PS exposure on stored RBCs, and if so whether this event was mediated by P2X7 or P2X1. Furthermore, this study aimed to determine if ATP-induced PS exposure on RBCs alters with *in vivo* or *ex vivo* aging.

MATERIALS AND METHODS

Materials

ATP, MRS2159 and adenine were from Sigma-Aldrich (St. Louis, MO). A438079 and AZ10606120 and (where indicated) NF499 were from Tocris Bioscience (Ellisville, MO). NF499 was from Cayman Chemical (Ann Arbor, MI). Ethidium bromide was from AMRESCO (Solon, OH).

Preparation of red blood cells

The ethics committees of the University of Wollongong and the Australian Red Cross Blood Service approved this project. Leukoreduced RBCs, from 36 randomly selected donors, were collected and prepared as described,[13] and stored in saline adenine glucose mannitol (SAGM) (Fresenius Kabi, Bad Homburg, Germany) at 2-6^{o}C. All experiments were performed using RBCs stored for less than 1 week (Week 1 RBCs) except when compared to RBCs stored for 6 weeks (Week 6 RBCs). Prior to use, RBCs were rejuvenated using a minimally invasive preconditioning protocol as recommended.[14] Briefly, 1 mL of RBCs in 15 mL preconditioning medium (145 mM NaCl, 5 mM KCl, 10 mM glucose, 0.5 mM $CaCl_2$, 0.1% bovine serum albumin and 10 mM HEPES, pH 7.4) were centrifuged (450 x *g* for 5 minutes). RBCs were then re-suspended in 15 mL preconditioning medium and incubated at room temperature for 1 hour. Finally, RBCs were centrifuged and washed twice with NaCl medium (147.5 mM NaCl, 2.5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4). In some experiments, rejuvenated RBCs were washed twice in KCl medium (150 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4), N-methyl-

D-glucamine (NMDG) Cl medium (147.5 mM NMDG Cl, 2.5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4) or Na gluconate medium (147.5 mM Na gluconate, 2.5 mM KCl, 5 mM glucose, 20 mM HEPES, pH 7.4) in place of NaCl medium. In other experiments, rejuvenated RBCs were fractionated according to cell density using Percoll (GE Healthcare, Uppsala Sweden) as previously described.[6]

Maintenance and preparation of RPMI-8226 cells

Human multiple myeloma RPMI-8226 cells were maintained as described.[15] Prior to use, cells were centrifuged (300 x *g* for 5 minutes) and washed twice with dye uptake medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM HEPES, pH 7.4).

Annexin V binding

ATP-induced PS exposure on RBCs was assessed as described.[8] Briefly, RBCs in NaCl medium (unless otherwise indicated) in 96-well U-bottom plates were incubated in the absence or presence of 1 mM ATP for 24 hours at 37^{o}C. In some experiments, RBCs were pre-incubated in the absence or presence of P2X antagonist (as indicated) for 15 minutes at 37^{o}C prior to ATP addition. Following ATP incubation, RBCs were gently re-suspended within the original wells, and 20 µL of RBCs were washed once in 1 mL Annexin V Binding Buffer (BioLegend, San Jose, CA) (450 X g for 3 minutes) and labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BioLegend) according to the manufacturer's instructions. Data was collected using a BD (San Jose, CA) LSR II flow cytometer and FACSDiva software, and the percentage of Annexin V^{+} cells (PS exposure) was determined using FlowJo software (Tree Star, Ashland, OR). In some experiments, the mean fluorescence intensity (MFI) of Annexin V binding on RBCs was determined using the geometric mean statistic of FlowJo software.

Ethidium$^+$ uptake

ATP-induced ethidium$^+$ uptake into RPMI 8226 cells was assessed as described.[15] Briefly, cells in dye uptake medium were pre-incubated in the absence or presence of P2X antagonist or adenine (as indicated) for 15 minutes at 37^{o}C. Ethidium$^+$ was added (25 μM), and cells were incubated in the absence or presence of ATP (as indicated) for a further 5 minutes at 37^{o}C. ATP incubations were stopped by addition of an equal volume of ice-cold $MgCl_2$ medium (145 mM NaCl, 5 mM KCl, 5 mM glucose, 20 mM $MgCl_2$, 10 mM HEPES, pH 7.4) and centrifugation. Data was collected using a LSR II flow cytometer and FACSDiva software, and the mean fluorescence intensity of ethidium$^+$ uptake was determined using FlowJo software.

Osmotic fragility

RBCs in NaCl medium (2% hematocrit) in 96-well U-bottom plates (200 μL/well) were incubated in the absence or presence of 1 mM ATP for 24 hours at 37^{o}C. Osmotic fragility of RBCs was then determined by lysis in buffered hypotonic saline as described.[16] Following ATP incubation, RBCs were gently re-suspended within the original wells, and 90 μL of RBCs from each well incubated with 180 μL of buffered hypotonic saline solutions (to give final concentrations of 0.35-0.6% (w/v) NaCl) or 180 μL of MilliQ water (100% lysis control) in 96-well U-bottom plates. RBCs were then incubated for 30 minutes at room temperature. Plates were centrifuged (1000 x *g* for 5 minutes) and supernatants measured at a wavelength of 540 nm in a Molecular Devices (Sunnyvale, CA) Spectramax Plus 384 microplate reader. Percent lysis was determined using the 100% lysis control.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using Prism 5 for Mac OS X (GraphPad Software, San Diego, CA). Differences between two, or three or more groups were compared using a paired Student's t-

test, or a one-way analysis of variance (using Tukey's multiple comparison test), respectively. The relationship between ATP-induced PS exposure and donor age at collection was compared using the Pearson correlation test. For all analyses $P < 0.05$ was considered significant.

RESULTS

P2X7 activation mediates PS exposure on stored RBCs

We have previously shown that extracellular ATP via P2X7 activation induces PS exposure on freshly isolated human RBCs.[8,9] Therefore, to determine if incubation with extracellular ATP also induces PS exposure on RBCs stored for less than 1 week, RBCs from 36 different donors were incubated in the absence or presence of ATP for 24 hours, and the percentage of PS exposure on RBCs determined by Annexin V binding and flow cytometry. Incubation in the absence of ATP resulted in a mean PS exposure of 1.2 ± 0.1% (range 0.4-3.4%). ATP incubation caused a five-fold increase in the mean PS exposure to 5.3 ± 0.5% (range 0.4-13.6%; $P < 0.0001$) (Fig. 1A). Notably, ATP-induced PS exposure was variable between donors (mean of 4.1 ± 0.5%, range 0.0-12.3%), with <1% ATP-induced PS exposure on RBCs from five donors and >10% ATP-induced PS exposure on RBCs from two other donors (Fig. 1B). ATP-induced PS exposure did not correlate with donor age, nor did ATP-induced PS exposure differ between female and male donors, or ABO or Rh blood groups (results not shown).

To determine if ATP-induced PS exposure was mediated by P2X7, stored RBCs were pre-incubated with two known P2X7 antagonists, A438079 and AZ10606120, before incubation with ATP for 24 hours. ATP incubation resulted in a four-fold increase in PS exposure compared to RBCs incubated in the absence of ATP (Fig. 2A). Pre-incubation with either A438079 or AZ10606120 significantly impaired ATP-induced PS exposure by an average of 96% ($P < 0.01$) and 99% ($P < 0.001$), respectively (Fig. 2A). PS exposure was

similar on RBCs incubated in the absence of both ATP and antagonist compared to RBCs incubated with antagonist alone (Fig. 2A).

To determine if P2X1, which is also present on human RBCs,[10,11] may contribute to ATP-induced PS exposure on RBCs, stored RBCs were pre-incubated with two known P2X1 antagonists, MRS2159 and NF499, before incubation with ATP for 24 hours. A438079 was included as a positive control. Again, ATP incubation resulted in a five-fold increase in PS exposure and pre-incubation with A438079 significantly impaired this process by an average of 98% ($P < 0.05$; Fig. 2B). Pre-incubation with MRS2159 also significantly impaired ATP-induced PS exposure by an average of 94% ($P < 0.05$; Fig. 2B). In contrast, pre-incubation with NF499 failed to impair ATP-induced PS exposure ($P > 0.05$; Fig. 2B). Similar results were observed with NF499 obtained from a second manufacturer (Tocris Bioscience) using RBCs from four different donors (results not shown). PS exposure was similar on RBCs incubated in the absence of ATP and antagonist compared to RBCs incubated with either antagonist alone (Fig. 2B).

To explore the possibility that MRS2159 but not NF499 impairs P2X7, RPMI 8226 cells, which express high amounts of functional P2X7 but negligible P2X1,[15] were pre-incubated with increasing concentrations of either MRS2159 or NF499, and the ATP-induced uptake of ethidium$^+$ assessed by flow cytometry. A438079 was included as a positive control. Similar to previous findings,[17] A438079 impaired ATP-induced ethidium$^+$ uptake in a concentration-dependent manner with a half maximal inhibitory concentration (IC_{50}) of 668 ± 40 nM (Fig. 2C). MRS2159 also impaired ATP-induced ethidium$^+$ uptake concentration-dependent manner, but with an IC_{50} of 40 ± 5 nM (Fig. 2C). In contrast, NF499, up to a concentration of 10 μM, failed to impair ATP-induced ethidium$^+$ uptake (Fig. 2C).

P2X7-mediated PS exposure on stored RBCs is not dependent on K^+, Na^+ or Cl^- fluxes

P2X7-mediated PS exposure on RBCs does not require Ca^{2+} influx,[9] however K^+, Na^+ and Cl^- fluxes can participate in stress-induced PS exposure on RBCs.[18-20] Therefore, to examine the role of these ion fluxes in P2X7-mediated PS exposure on RBCs, cells were re-suspended in either NaCl, KCl, NMDG Cl or Na gluconate medium and the ATP-induced PS exposure was assessed as above. KCl medium prevents the net loss of intracellular K^+, while NMDG Cl and Na gluconate media are nominally free of Na^+ and Cl^- ions, respectively, and thus prevent the respective net influx of Na^+ or Cl^- ions. As above, ATP induced a five- to six-fold increase in PS exposure on RBCs suspended in NaCl medium compared to RBCs in NaCl medium incubated in the absence of ATP (Fig. 3A,B). ATP induced a eight-fold, five-fold and seven-fold increase in PS exposure on RBCs suspended in KCl ($P < 0.01$), NMDG Cl ($P < 0.05$) or Na gluconate ($P < 0.001$) medium, respectively, compared to RBCs in corresponding medium incubated in the absence of ATP (Fig. 3A,B). PS exposure on RBCs incubated in the absence of ATP was similar between the four different media (Fig. 3A,B).

Adenine does not impair P2X7 activation

Due to structural similarities, ATP derivatives may potentially impair P2X7 activation by binding to the ATP-binding site. Notably, SAGM and SAGM2 additive solutions contain approximately 485 and 910 μM adenine, respectively. Thus, there is a possibility that adenine may prevent P2X7 activation on RBCs during storage. To determine if adenine can impair P2X7 activation, RPMI 8226 cells were pre-incubated with either 485 or 910 μM adenine, followed by incubation with increasing ATP concentrations and ethidium$^+$ uptake was assessed as above. As repeatedly observed with this cell line,[15,21] incubation with ATP alone induced ethidium$^+$ uptake in a concentration-dependent manner with a half maximal effective concentration (EC_{50}) of 104 ± 7 μM and near-maximal uptake at 0.3 mM (Fig. 4). Similar results were observed following pre-incubation with either 485 or 910 μM adenine, with an

EC_{50} of 99 ± 11 μM and 103 ± 9 μM, respectively and near-maximal ethidium$^+$ uptake at 0.3 mM ATP at either adenine concentration (Fig. 4). Basal ethidium$^+$ uptake in the presence of adenine was similar to basal ethidium$^+$ uptake in the absence of adenine (results not shown). Additionally, RPMI 8226 cells were pre-incubated with increasing concentrations of adenine, followed by incubation with 120 μM ATP and the ethidium$^+$ uptake assessed as above. Pre-incubation of RPMI 8226 cells with adenine up to concentrations of 3 mM did not alter ATP-induced ethidium$^+$ uptake (results not shown).

Extracellular ATP does not alter osmotic fragility of stored RBCs

Extracellular ATP, especially via P2X7 activation, induces various membrane-related changes in a number of cell types.[22] Therefore, to examine if ATP incubation altered the osmotic fragility (membrane resistance) of stored RBCs, cells were incubated in the absence or presence of ATP, and then the osmotic fragility of these cells was assessed by lysis in hypotonic saline solutions and spectrophotometry. As expected, increasing hypotonicity induced the lysis of RBCs following incubation in the absence of ATP in a concentration-dependent manner and with 50% of lysis occurring at a saline concentration of 0.39 ± 0.01% (Fig. 5). Near identical results were observed with RBCs following ATP incubation, with increasing hypotonicity inducing lysis in a concentration-dependent manner and with 50% of lysis also occurring at a saline concentration of 0.39 ± 0.01% (Fig. 5).

P2X7 activation mediates PS exposure on RBCs aged *in vivo* or *ex vivo*

To determine if the amount of P2X7-mediated PS exposure changes during the lifespan of RBCs *in vivo*, RBCs were separated into four fractions, using discontinuous Percoll density centrifugation, enriched with RBCs of various ages. Fractions 1 and 4 represent younger and older RBCs, respectively, while Fractions 2 and 3 represent RBCs of intermediate ages.[23] Unfractionated and fractionated RBCs were then incubated in the absence or presence of ATP,

and PS exposure was assessed as above. ATP induced a four-fold increase in PS exposure on unfractionated RBCs compared to unfractionated RBCs incubated in the absence of ATP ($P < 0.001$; Fig. 6). Increases in ATP-induced PS exposure of a similar magnitude were observed on RBCs in each of the four fractions ($P < 0.001$; Fig. 6). PS exposure was similar between unfractionated RBCs and RBCs in each fraction incubated in the absence of ATP (Fig. 6). Similar results between unfractionated and fractionated RBCs were observed when ATP-induced PS exposure was examined as MFI of Annexin V binding (results not shown).

Finally, to determine if the amount of P2X7-mediated PS exposure changes on RBCs aged *ex vivo*, ATP-induced PS exposure was assessed on RBCs stored for less than 1 week (Week 1 RBCs) and for 6 weeks (Week 6 RBCs). ATP induced a mean four-fold increase in PS exposure on Week 1 RBCs compared to Week 1 RBCs incubated in the absence of ATP ($P < 0.001$; Fig. 7A). Similarly, ATP induced a mean four-fold increase in PS exposure on Week 6 RBCs compared to Week 6 RBCs incubated in the absence of ATP ($P < 0.001$; Fig. 7A). Further, mean ATP-induced PS exposure was similar between Week 1 RBCs and Week 6 RBCs ($P = 0.7034$; Fig. 7B). ATP-induced PS exposure on RBCs from most donors decreased or increased slightly over the 6-weeks storage period, however these changes did not exceed a 50% reduction or two-fold increase, respectively, with the exception of RBCs from one donor in which a 2-fold increase was observed (Fig. 7B). Similar results between Week 1 and Week 6 RBCs were observed when ATP-induced PS exposure was examined as MFI of Annexin V binding (results not shown).

DISCUSSION

P2X1 and P2X7 are the only reported functional P2X receptors on human RBCs.[10,24,25] Further, P2X7 activation is known to induce PS exposure on freshly isolated human RBCs.[8,9] Using stored human RBC concentrates the current study demonstrates that ATP induces PS exposure on stored RBCs and that this process is mediated by P2X7 but not P2X1. ATP-

induced PS exposure on stored RBCs was near-completely inhibited by two structurally unrelated P2X7 antagonists, A438079 and AZ10606120,[26,27] but not by the P2X1 antagonist, NF499.[28] ATP-induced PS exposure on RBCs was also impaired by MRS2159, which is generally considered a P2X1 antagonist.[29] However, measurements of ATP-induced ethidium$^+$ uptake into human RPMI 8226 cells demonstrated that MRS2159 is also a potent antagonist of P2X7 with an IC_{50} at least one log lower than that of A438079. Notably, MRS2159, but not NF449, has also been shown to impair ATP-induced Ca^{2+} fluxes in HEK-293 cells expressing heterologous P2X7.[30] Collectively, these two findings support the concept that MRS2159 is also an antagonist of P2X7 and that ATP-induced PS exposure on stored RBCs is mediated by P2X7 and not P2X1. The absence of a role for P2X1 in ATP-induced PS exposure on stored RBCs, is consistent with our previous findings in which adenosine 5'-diphosphate, an agonist of P2X1 but not P2X7,[31] failed to induce PS exposure on freshly isolated RBCs.[9] Thus, ATP-induced PS exposure in human RBCs appears to be exclusively mediated by P2X7. In contrast, both P2X1 and P2X7 may be involved in other signaling events in human RBCs such as α-hemolysin-, leukotoxin- and complement-induced hemolysis of human RBCs.[10,32-34]

ATP-induced PS exposure was similar between fractions enriched with RBCs of various ages. These four fractions contained RBCs of increasing density, a useful but imprecise marker of cell age.[23] Thus, the current study demonstrates that the amount of P2X7-mediated PS exposure on human RBCs does not significantly change during *in vivo* aging. These results contrast findings with hyperosmotic stress-induced PS exposure on fractionated RBCs.[6] In this prior study, RBCs fractions, isolated in the same manner as the current study, demonstrated that increased cell density was associated with increased susceptibility to stress-induced PS exposure. Similarly, this same group, using elutriation followed by discontinuous density gradient centrifugation, demonstrated that increased cell age was associated with increased susceptibility to PS exposure induced by energy (glucose) depletion.[35] On average, ATP-induced PS exposure was also similar between RBCs stored for less than 1 week and for 6

weeks. During storage, RBCs undergo a number of biochemical and structural changes that parallel that of aging RBCs *in vivo* and as such RBCs are also considered to age *ex vivo*.[4] Thus, the current study demonstrates that the amount of P2X7-mediated PS exposure on human RBCs does not significantly change during *ex vivo* aging. Again these results contrast findings with hyperosmotic stress-induced PS exposure on stored RBCs.[6] In this previous study, RBCs demonstrated that increased storage time was associated with increased susceptibility to stress-induced PS exposure.

The reasons for the differences between stress-induced and P2X7-mediated PS exposure in aged RBCs in the previously mentioned studies and the current study remain unknown, but may relate to differences in the signaling pathways involved. P2X7-mediated PS exposure on RBCs is independent of Ca^{2+} influx,[9] while stress-induced PS exposure requires Ca^{2+} influx.[7] Moreover, stress-induced PS exposure on RBCs is partly dependent on K^+ and Cl^- fluxes, while Na^+ influx may also play a role in this process in these cells.[18-20] In contrast, using medium either nominally free of Na^+ or Cl^-, or medium containing a high concentration of K^+, the current study was unable to demonstrate a role for Na^+ or Cl^- influx or K^+ efflux in P2X7-mediated PS exposure on RBCs. The increase in ATP-induced PS exposure seen in RBCs incubated in either KCl or NMDG Cl medium compared to NaCl medium, is consistent with the well-known inhibitory role of Na^+ on P2X7 activation.[36]

Using RBC concentrates from 36 different donors the current study demonstrates that the amount of P2X7-mediated PS exposure varies between individuals. This variability is consistent with observed variation in other P2X7-mediated events including ethidium$^+$ uptake into leukocytes, which is predominately explained by single nucleotide polymorphisms in the *P2RX7* gene that alter receptor function.[37,38] Thus, the variability observed in P2X7-mediated PS exposure on stored RBCs between donors is most likely due to loss- or gain-of-functions polymorphisms in the *P2RX7* gene. In support of this, we have previously shown that loss- or gain-of-functions polymorphisms alter RBC P2X7 function accordingly,[24,39] including ATP-

induced PS exposure in freshly isolated human RBCs.[9] Due to logistical and ethical constraints in this study it was not possible to collect DNA from the volunteer blood donors. Thus, the *P2RX7* genotype of all donors in the current study remains unknown. Of note, PS exposure on stored RBCs may serve as parameter for donor-dependent variation in the quality of RBC concentrates.[40] Therefore, it remains possible that *P2RX7* genotype and the propensity of RBCs to undergo P2X7-mediated PS exposure may contribute to this donor-dependent variation in RBC product quality.

The physiological role of P2X7-mediated PS exposure on human RBCs remains to be elucidated. Given that this process does not increase during RBC aging (this study) and that direct evidence for PS exposure in the removal of senescent RBCs is lacking,[1] it appears unlikely that P2X7-mediated PS exposure plays a major role in the removal of healthy, senescent RBCs. Rather it remains possible that ATP-induced PS exposure may act as a marker of recent P2X7 activation and that some other P2X7-mediated event, such as the ATP-induced release of epoxyeicosatrienoic acids (as observed with rat RBCs),[41] is the main physiological role of P2X7 activation in human RBCs. Alternatively, given that P2X7 transcripts are found in human RBC progenitors[42] and that relatively high amounts of functional P2X7 are found in a murine RBC progenitor cell line,[43,44] this receptor may serve an important role in erythropoiesis. If so, P2X7 on mature RBCs may be an evolutionary or functional relic, as proposed for other RBC membrane proteins.[45,46] However it has been recently argued that so-called relic channels, specifically the Gardos channel and voltage-dependent anion channel, may serve important roles in RBC homeostasis.[46] Thus, a potential role for P2X7 in RBC homeostasis cannot be excluded at this time. Finally, it needs to be considered that P2X7 on RBCs may be important in certain pathophysiological states, even in the absence of a role for this receptor in healthy RBCs. P2X7 along with P2X1 are thought to be important in facilitating bacterial toxin- and complement-induced hemolysis of human RBCs.[10,32-34] Additionally, P2X7 activation is deemed to allow malarial parasites to invade or

escape RBCs.[47,48] Thus, given the presence of functional P2X7 on stored RBCs, RBC concentrates may provide a readily available source to further elucidate the physiological and pathophysiological roles of P2X7 in human RBCs.

In conclusion, the current study indicates that the propensity of RBCs to undergo P2X7-mediated PS exposure does not alter during *in vivo* and *ex vivo* aging. This suggests that P2X7 activation is unlikely to be a common mechanism in the removal of senescent RBCs or stored RBCs following transfusion.

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CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Fig. 1. ATP induces PS exposure on stored RBCs. (A, B) Week 1 RBCs in NaCl medium were incubated in the absence or presence of 1 mM ATP for 24 hours at 37°C. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. (A) Each symbol represents the percentage of Annexin V^+ RBCs, from 36 individual donors, following incubation in the absence (basal) or presence of ATP; ****$P < 0.0001$ compared to basal. (B) Each symbol represents the percentage of ATP-induced PS exposure RBCs, from the 36 individual donors, determined as the difference in the percentage of Annexin V^+ RBCs following incubation in the presence and absence of ATP; bar represents group mean.

Fig. 2. P2X7 antagonists impair ATP-induced PS exposure on stored RBCs. (A, B) Week 1 RBCs in NaCl medium were pre-incubated for 15 minutes in the absence or presence of (A, B) 10 μM A438079, (A) 1 μM AZ10606120 or (B) 10 μM MRS2159 or 10 μM NF499, and (A, B) then in the absence (basal) or presence of 1 mM ATP for 24 hours at 37°C. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. (C) RPMI 8226 cells in dye uptake medium were pre-incubated for 15 minutes in the absence or presence of A438079, MRS2159 or NF499 (as indicated). Ethidium$^+$ (25 μM) was then added and the cells incubated in the absence or presence of 120 μM ATP (approximate to the EC_{50} for human P2X7)[15] for 5 minutes at 37°C. Incubations were stopped by addition of $MgCl_2$ medium and centrifugation, and the mean fluorescence intensity of ethidium$^+$ uptake determined by flow cytometry. (A, B) Results are mean ± SEM (A, n = 3 individual donors; B, n = 4 individual donors); *$P < 0.05$ or ***$P < 0.001$ compared to corresponding basal, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ or $^{\dagger\dagger\dagger}P < 0.001$ compared to corresponding ATP alone. (C) Results are percent maximum response to ATP in the absence of antagonist (mean ± SEM, n = 3 experiments).

Fig. 3. P2X7-mediated PS exposure on stored RBCs is not dependent on K^+, Na^+ or Cl^- fluxes. Week 1 RBCs in (A, B) NaCl, (A), KCl, NMDG Cl or (B) Na gluconate medium were incubated in the absence (basal) or presence of 1 mM ATP for 24 hours at 37°C. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. Results are mean ± SEM (A, n = 5 individual donors; B, n = 4 individual donors studied in triplicate); $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$ compared to corresponding basal.

Fig. 4. Adenine does not impair P2X7 activation. RPMI 8226 cells in dye uptake medium were pre-incubated for 15 minutes in the absence (control) or presence of 485 μM or 910 μM adenine. Ethidium$^+$ (25 μM) was then added and the cells incubated in the absence or presence of ATP (as indicated) for 5 minutes at 37°C. Incubations were stopped by addition of $MgCl_2$ medium and centrifugation, and the mean fluorescence intensity of ethidium$^+$ uptake determined by flow cytometry. Results are percent maximum response to 1 mM ATP alone (mean ± SEM, n = 3 experiments).

Fig. 5. Extracellular ATP does not alter osmotic fragility of stored RBCs. Week 1 RBCs in NaCl medium were incubated in the absence or presence of 1 mM ATP for 24 hours at 37°C. Cells were then incubated in buffered hypotonic saline (as indicated) for 30 minutes at room temperature, and the absorbance of supernatants measured by spectrophotometry. Results are percent lysis compared 100% lysis control (n = 3 individual donors).

Fig. 6. P2X7 activation mediates PS exposure on RBCs aged *in vivo*. Week 1 RBCs were separated by discontinuous Percoll density centrifugation into four fractions of increasing density (Fractions 1-4, respectively). Unfractionated and fractionated RBCs in NaCl medium were then incubated in the absence or presence of 1 mM ATP for 24 hours at 37°C. Cells were

labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. Results are mean ± SEM (n = 3 individual donors); ***$P < 0.001$ compared to corresponding basal.

Fig. 7. P2X7 activation mediates PS exposure on RBCs aged *ex vivo*. (A, B) Week 1 and Week 6 RBCs in NaCl medium were incubated in the absence or presence of 1 mM ATP for 24 hours at 37^{o}C. Cells were labeled with FITC-conjugated Annexin V and analyzed by flow cytometry. (A) Results are mean ± SEM (n = 15 individual donors); ***$P < 0.001$ compared to corresponding basal. (B) Each symbol represents the percentage of ATP-induced PS exposure RBCs, from the 15 individual donors, determined as the difference in the percentage of Annexin V^{+} RBCs following incubation in the presence and absence of ATP.

Figure 1

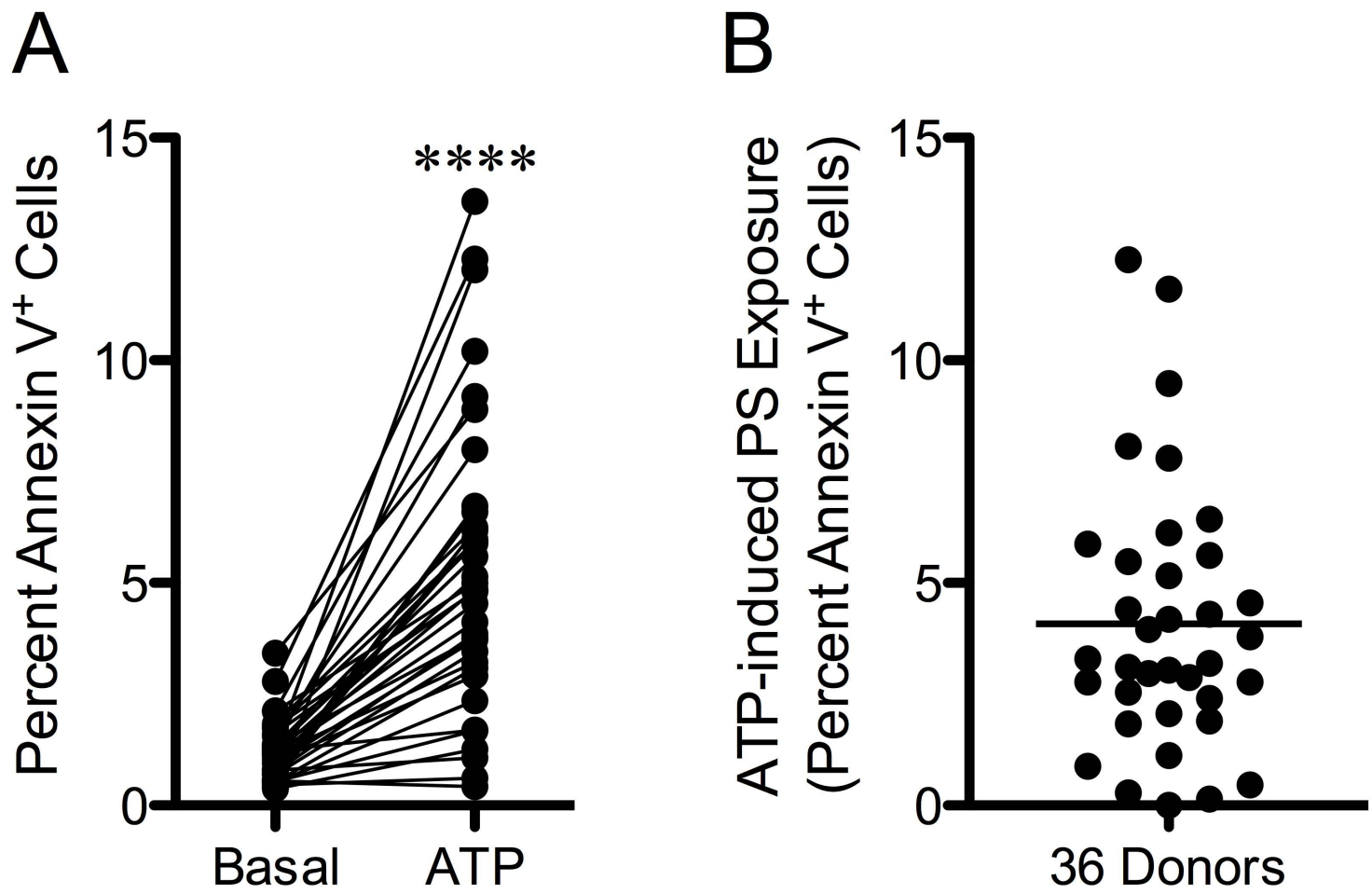

A
B
Percent Annexin V+ Cells
15
10
5
0
****
Basal
ATP
ATP-induced PS Exposure
(Percent Annexin V+ Cells)
15
10
5
0
36 Donors

Figure 2

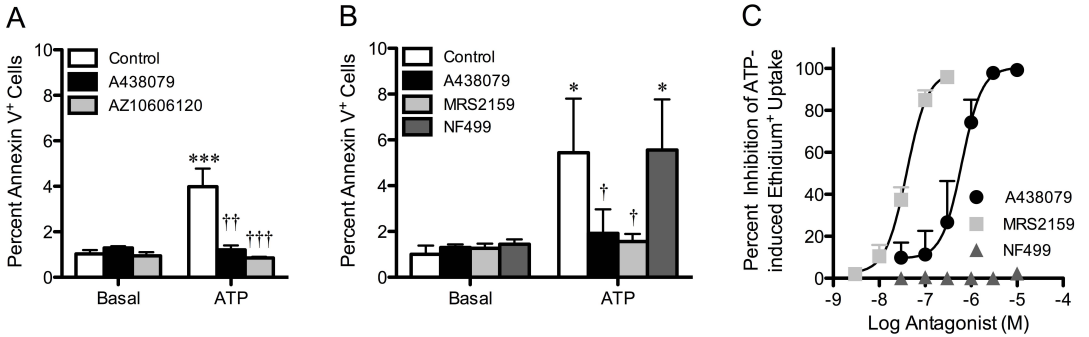

A
Percent Annexin V+ Cells
Control
A438079
AZ10606120
***
††
†††
Basal
ATP
B
Percent Annexin V+ Cells
Control
A438079
MRS2159
NF499
*
†
†
*
Basal
ATP
C
Percent Inhibition of ATP-induced Ethidium+ Uptake
A438079
MRS2159
NF499
Log Antagonist (M)

Figure 3

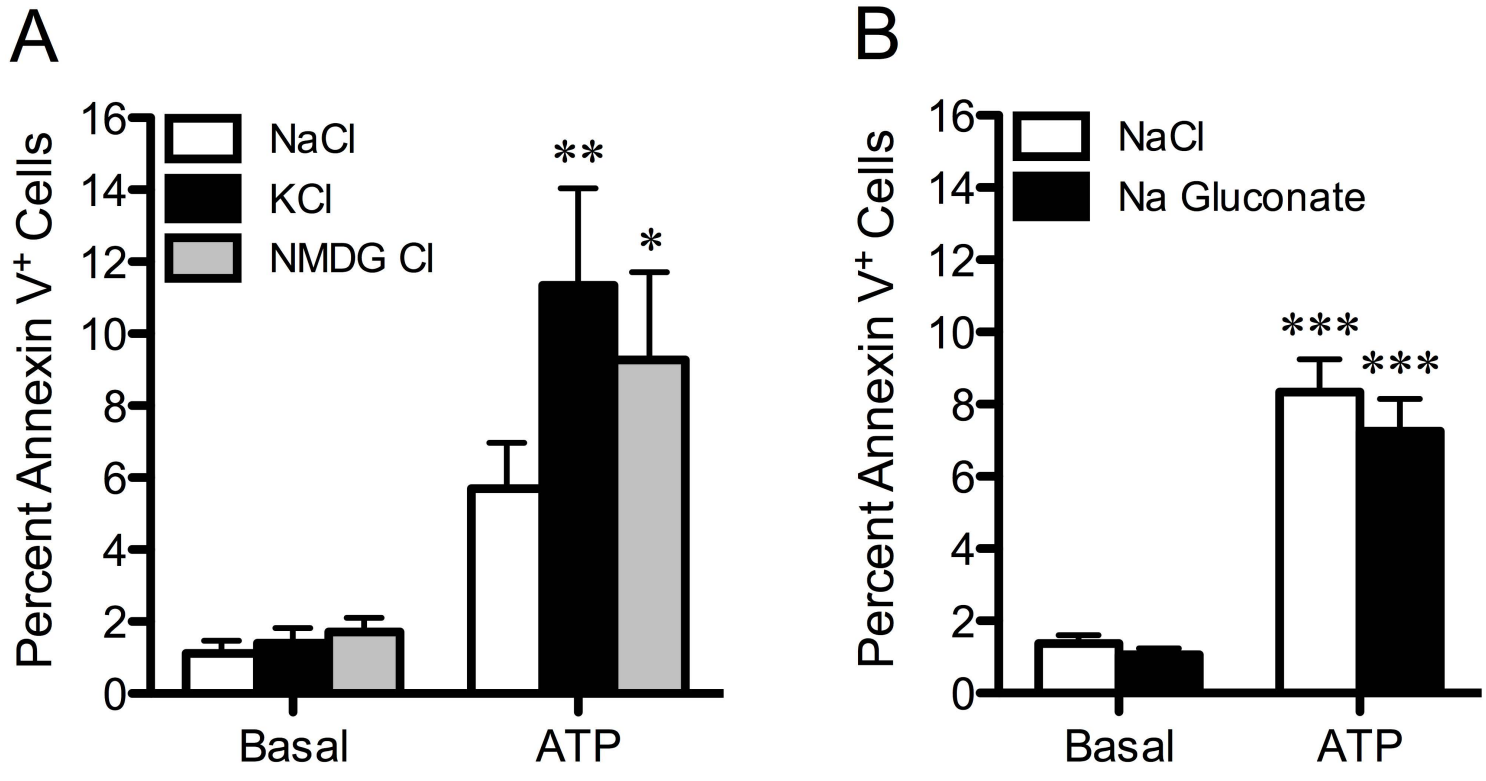

A
Percent Annexin V+ Cells
16
14
12
10
8
6
4
2
0
NaCl
KCl
NMDG Cl
**
*
Basal
ATP
B
Percent Annexin V+ Cells
16
14
12
10
8
6
4
2
0
NaCl
Na Gluconate
***
***
Basal
ATP

Figure 4

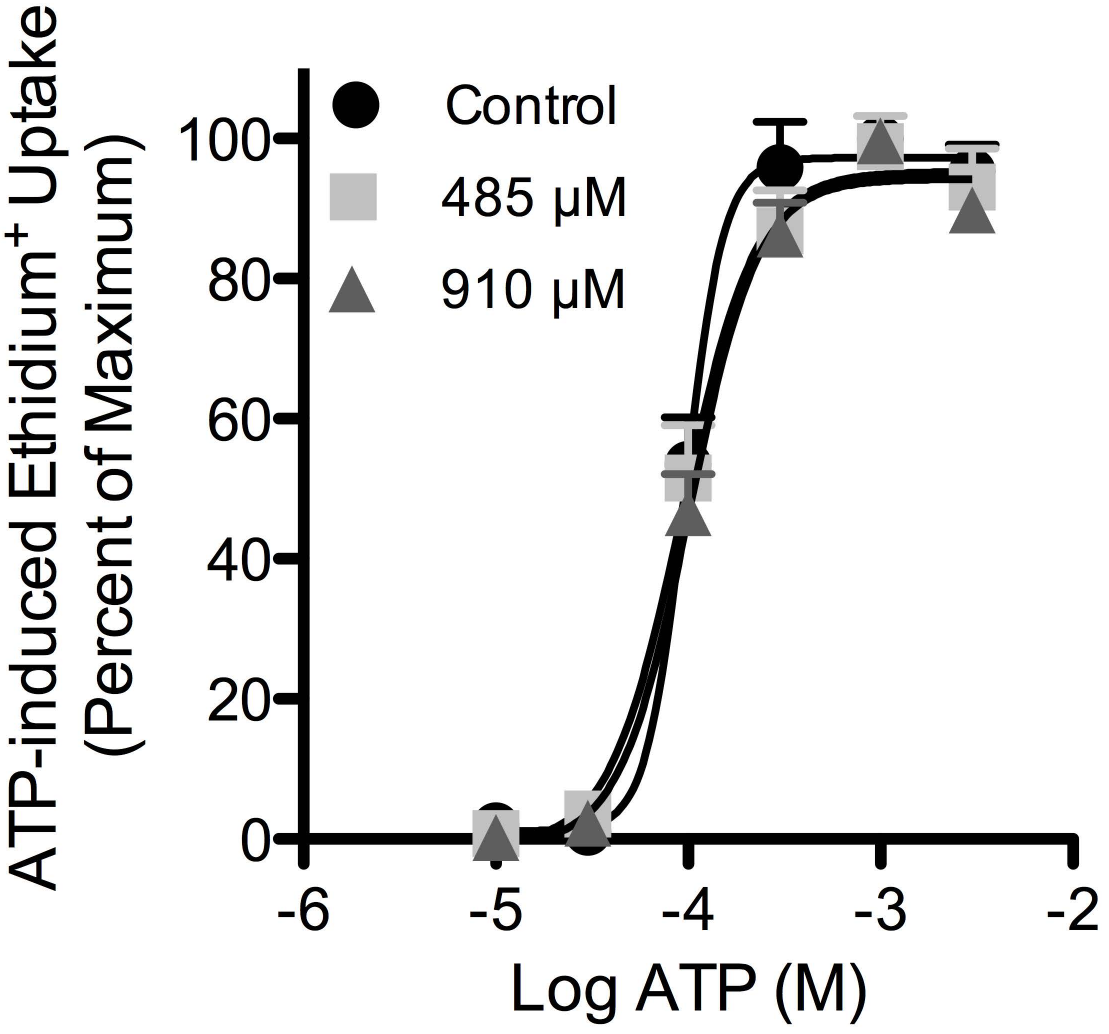

ATP-induced Ethidium+ Uptake
(Percent of Maximum)
100
80
60
40
20
0
Control
485 µM
910 µM
-6
-5
-4
-3
-2
Log ATP (M)

Figure 5

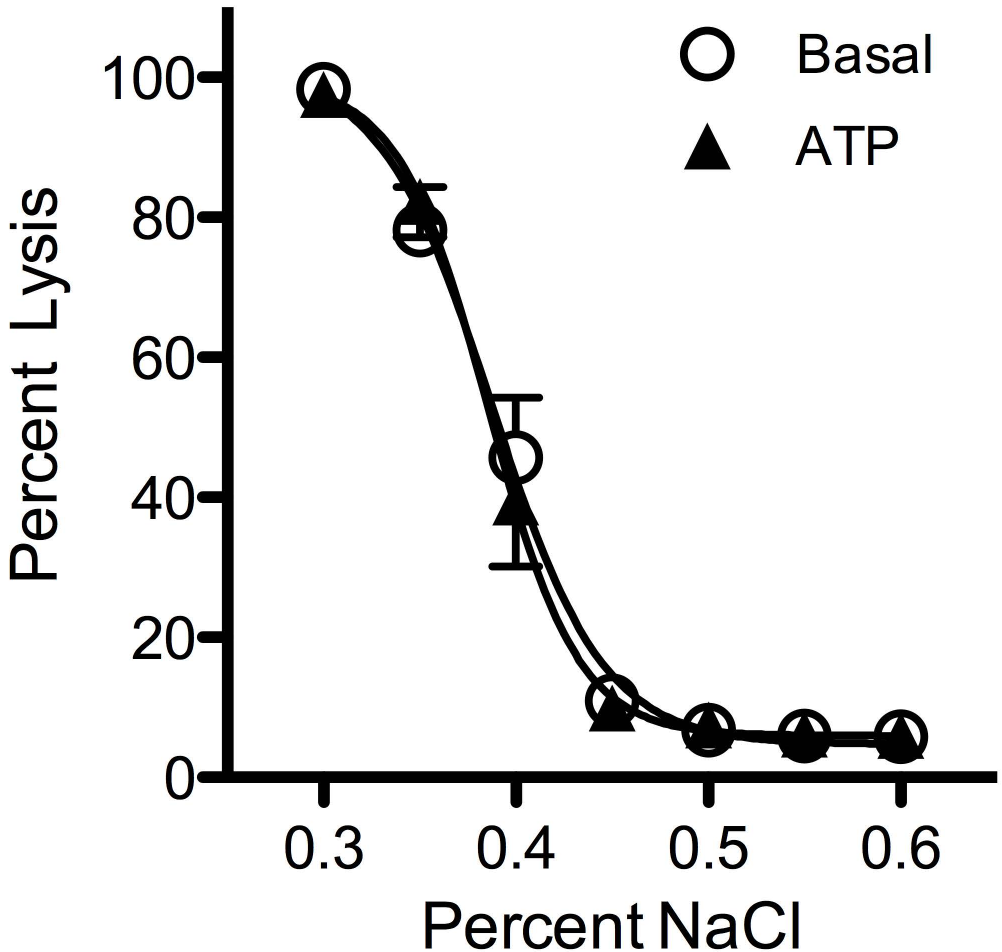

Basal
ATP
100
80
60
40
20
0
Percent Lysis
0.3
0.4
0.5
0.6
Percent NaCl

Figure 6

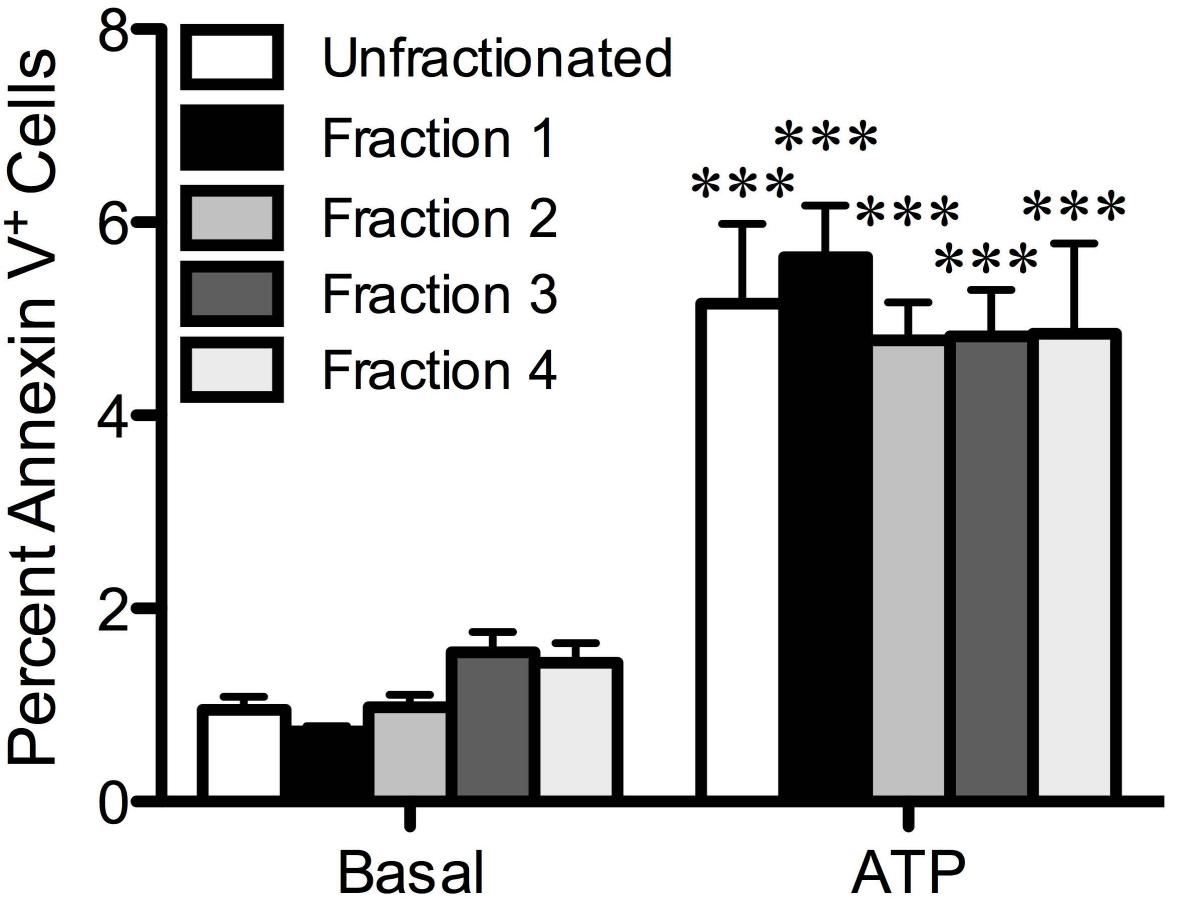
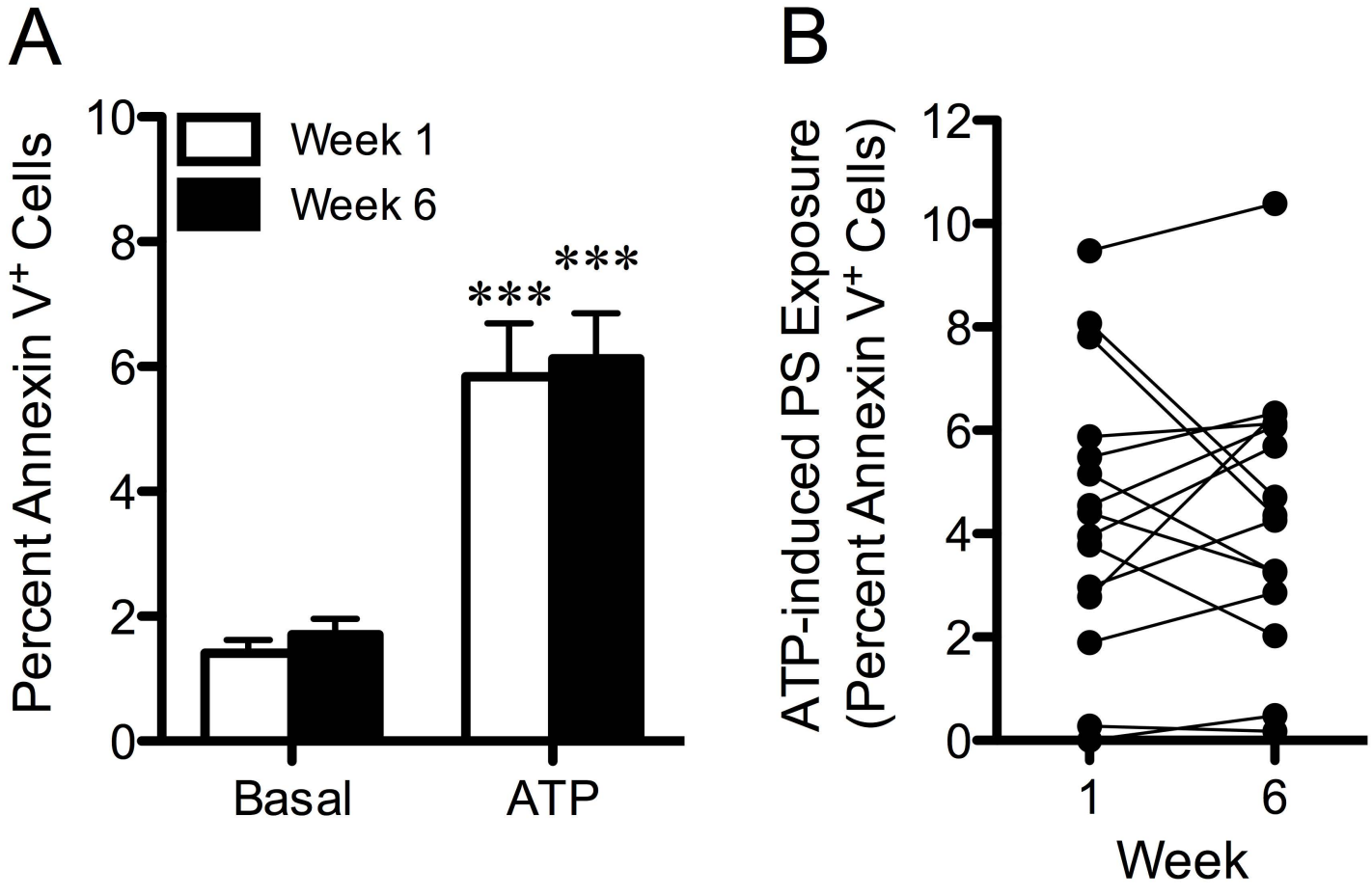

Percent Annexin V+ Cells
8
6
4
2
0
Unfractionated
Fraction 1
Fraction 2
Fraction 3
Fraction 4
***
***
***
***
***
Basal
ATP

Figure 7


A
Percent Annexin V+ Cells
10
8
6
4
2
0
Week 1
Week 6
***
***
Basal
ATP
B
ATP-induced PS Exposure
(Percent Annexin V+ Cells)
12
10
8
6
4
2
0
1
6
Week